Cancer Chemother Pharmacol (1998) 41: 353–360

ORIGINAL ARTICLE

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Reduced activity of topoisomerase II in an Adriamycin-resistant human stomach-adenocarcinoma cell line

Received: 1 June 1997 / Accepted: 28 August 1997

Abstract A human stomach-adenocarcinoma cell line (MKN-45) was selected for resistance to Adriamycin by stepwise exposure to increasing concentrations of this agent. The resulting cell line (MKN/ADR) exhibited a high level of cross-resistance to topoisomerase II (topo II)-targeted drugs such as Adriamycin, mitoxantrone, and etoposide but showed no cross-resistance to other chemotherapeutic agents such as cisplatin, carboplatin, 5-fluorouracil, or mitomycin-C. P-glycoprotein encoded by the *mdr-1* gene was not overexpressed in the MKN/ADR cell line. The doubling time of the MKN/ADR cell line (2.1 days) increased only slightly as compared with that of the MKN cell line (1.7 days). The patterns of cross-resistance to various chemotherapeutic agents led us to examine the cellular contents of topo II in both the drug-sensitive and the drug-resistant cells. Extractable topo II enzyme activity was 3-fold lower in MKN/ADR cells as compared with the parental MKN cells. Levels of topoisomerase I (topo I) catalytic activity were similar in both wild-type MKN and drug-resistant MKN/ADR cells. Southern-blot analysis of genomic DNA probed with topo IIα or IIβ showed no sign of either gene rearrangement or hypermethylation. Northern-blot analysis revealed that both topo IIα and topo IIβ mRNA transcripts were essentially identical in the MKN and MKN/ADR cells. In contrast, Western-blot analysis revealed an approximately 20-fold lower level of topo IIα in drug-resistant cells as compared with drug-sensitive cells, whereas topo IIβ levels were similar in both lines. Moreover, the amount of in vivo topo IIα-DNA covalent complexes formed in the presence of etoposide was also approximately 20-fold lower in drug-resistant cells. No mutation was detected in the promoter region of the topo IIα gene in resistant cells as compared with sensitive cells. Thus, low levels of topo IIα polypeptide cannot be ascribed to changes in the mRNA levels. Collectively, the data suggest that a quantitative reduction in topo IIα may contribute to the resistance of MKN cells to Adriamycin and other topo II-targeted drugs.

Key words Topoisomerase II · Anticancer drug · Adriamycin · Etoposide · Covalent cleavable complex

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Introduction

Eukaryotic DNA topoisomerases are nuclear enzymes that function in almost all aspects of DNA metabolism, including replication, transcription, recombination, repair, and chromosomal condensation, by controlling the topological state of DNA [27]. Type II DNA topoisomerases (topo II) are ATP-dependent enzymes that catalyze DNA strand passage through transient double-strand breaks in the DNA [20]. There are two isoforms of human topo II that have been designated as topo IIα and topo IIβ, which are encoded by genes located on chromosomes 17 and 3, respectively [4, 24]. These isoforms exist as homodimers and their amino acid sequences show homology at regions believed to be

functionally significant [4]. Topo IIα activity is thought to be more sensitive to topo II-active agents relative to the topo IIβ form [8]. Topo IIα and IIβ isoforms, however, differ in other key biochemical and biophysical properties, suggesting that their roles in the cell may not be the same [8]. The expression of topo IIα varies during the cell cycle. It is low in quiescent cells but maximal in the G2-M phase, whereas the level of topo IIβ remains constant throughout the cell cycle [28].

Clinically, these enzymes are the cellular targets of important antitumor drugs that stabilize double-strand breaks and topo II-DNA covalent complexes [13]. Many topo II poisons are DNA intercalators such as Adriamycin, daunorubicin, ellipticine, and amsacrine (*m*-AMSA), and few are nonintercalators such as etoposide (VP-16) and teniposide (VM-26) [13]. In tumor cells selected for resistance to topo II-targeted drugs, the most common mechanism of drug resistance involves reduced formation of cleavable complexes due to the expression of decreased amounts or activities of topo IIα [16] or topo IIβ [9]. Although the relationship between the topo II level and drug sensitivity in tumor cells expressing altered topo II has been described, many questions about the role of topo II in the development of multidrug resistance remain.

Adriamycin, an anthracycline antitumor agent, is clinically active against a variety of human malignancies. Several mechanisms have been proposed to explain the antitumor activity of Adriamycin, including DNA damage mediated by topo II [25], interaction with membranes [26], and generation of oxygen free radicals [21]. Previous studies with Adriamycin-resistant cells have suggested that cellular resistance to Adriamycin is correlated with quantitative and qualitative changes in topo II enzyme activity [6, 14]. In the present study we isolated an Adriamycin-resistant human stomach-adenocarcinoma cell line (MKN/ADR) by exposure to increasing amounts of the drug. The sensitivity of the MKN cells and the MKN/ADR cells to various antitumor agents was correlated with their levels of expression of the two isoforms, topo IIα and topo IIβ.

Materials and methods

Cell lines

The MKN-45 human stomach-adenocarcinoma cell line was kindly provided by Dr. N. Saijo (National Cancer Center Research Institute, Tokyo, Japan). Cells were propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a balanced air humidified incubator with an atmosphere containing 5% CO_2.

Establishment of an Adriamycin-resistant cell line

An Adriamycin-resistant subline was derived by continuous exposure of MKN-45 cells to Adriamycin (Sigma) at concentrations starting at 0.01 μg/ml and increasing in a stepwise manner to 0.6 μg/ml. Cell lines capable of sustained growth in medium containing Adriamycin were considered to be resistant after 3 months. The Adriamycin-resistant subline is referred to as MKN/ADR hereafter. MKN/ADR cells were challenged monthly with Adriamycin at 0.6 μg/ml and, after their maintenance in drug-free medium for 2–3 weeks, experiments with the resistant cells were performed. Cell viability was determined by trypan blue exclusion test.

Drug-sensitivity assay

The drug-sensitivity test was performed by the microculture tetrazolium (MTT) assay as previously described [1]. Cells were seeded at a density of 5×10^3 cells in a 96-well plate, which gave an optical density value in the range of 0.8–1.2 at 540 nm. Cells were treated with various concentrations of drugs and incubated for 4 days in the humidified CO_2 incubator. Each experiment was performed in triplicate. IC_{50} was defined as a 50% reduction in optical density.

Measurement of plating efficiency and doubling time

Soft agar colony-formation assays were performed as previously described [12]. Single-cell suspensions were diluted with RPMI-FBS containing 0.3% agar (cell density 1×10^3 cells/ml), and 1 ml of this mixture was overlayed onto the underlayer, which was prepared in a six-well plate with 0.5% agar in enriched McCoy's 5A medium (Gibco/BRL). Enriched McCoy's 5A medium consisted of 40 ml heat-inactivated FBS, 20 ml heat-inactivated horse serum, 4 ml 2.2% sodium pyruvate, 4 ml 200 m*M* glutamine, 0.8 ml 2.1% serine, and 4 ml penicillin (100 U/ml) and streptomycin (100 μg/ml) mixed with 400 ml McCoy's 5A medium. The cultures were incubated for 14 days. Colonies bigger than 60 μm in diameter were counted by Artek counter Model 880 (IPI International Inc., Virginia). Each experiment was performed in triplicate. For the measurement of doubling time, cells were seeded at three different concentrations (between 1×10^4 and 1×10^5 cells/ml) and viable cell numbers were counted by trypan blue exclusion test. The doubling time was calculated from the growth curve.

Preparation of cellular and nuclear extracts

Cells were extracted as previously described [6]. In brief, exponentially growing cells (2.5×10^5 cells/ml) were harvested by centrifugation and washed three times with ice-cold phosphate-buffered saline (PBS). Cell pellets were resuspended in extraction buffer A [10 m*M* TRIS-HCl (pH 7.5), 25 m*M* KCl, 1 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride (PMSF)] at 4 °C for 15 min, followed by vigorous pipetting at least 40 times with a Gilson P200 pipetman. The solution was then adjusted to 0.5 M NaCl by the addition of an equal volume of extraction buffer B [50 m*M* TRIS-HCl (pH 7.5), 1 m*M* ethylenediaminetetraacetic acid (EDTA), 1 *M* NaCl, 1 m*M* dithiothreitol, 1 m*M* PMSF] and gently stirred. After extraction for 2 h on ice, the mixture was centrifuged at 16 000 *g* for 20 min at 4 °C. The supernatant was saved for Western-blot analysis of gluthathione-*S*-transferase (GST)-π and GST-L and for measurement of superoxide dismutase (SOD) activity.

For the preparation of nuclear extracts, exponentially growing cells (2.5×10^5 cells/ml) were pelleted and washed three times with ice-cold PBS. The cell pellets were resuspended in 1 ml nucleus buffer [150 m*M* NaCl, 1 m*M* KH_2PO_4; 5 m*M* $MgCl_2$; 1 m*M* ethyleneglycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 0.2 m*M* dithiothreitol, 1 m*M* PMSF (pH 6.4)] on ice and then mixed with an additional 9 ml nucleus buffer containing 0.3% Triton X-100. The suspension was gently mixed by rotation for 10 min at 4 °C and centrifuged at 2000 *g* for 10 min at 4 °C. The nuclear pellet was washed once with Triton-free nucleus buffer, and nuclei were extracted in nucleus buffer containing 0.5 *M* NaCl for 30 min on ice. The mixture was then centrifuged at 16 000 *g* for 20 min at 4 °C and the supernatant was saved for measurement of topo I and topo II enzyme activities. Protein concentrations were

determined by the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.). The extracts were aliquoted and stored at −80 °C.

Topo I and topo II catalytic activity assay

Topo I activity was assayed by relaxation of supercoiled pBS DNA. The reactions were initiated by the addition of varying amounts of nuclear extracts to supercoiled DNA (0.2 μg) in a standard reaction mixture [50 m*M* TRIS-HCl (pH 7.5), 1 m*M* EDTA, 100 m*M* NaCl] for 30 min at 37 °C. Reactions were stopped by the addition of 0.1 vol. 10% sodium dodecyl sulfate (SDS). The DNA samples were then analyzed on a 1.2% native agarose gel. Topo II catalytic activity was assayed by the ATP-dependent decatination of kinetoplast DNA [3]. The standard reaction mixture for the decatenation assay contained 0.2 μg kDNA, 50 m*M* TRIS-HCl (pH 7.5), 120 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 0.5 m*M* ATP, and 30 μg bovine serum albumin (BSA) per ml. The reactions were incubated for 15 min at 37 °C and terminated with 0.1 vol. stop buffer (5% Sarkosyl, 0.025% bromophenol blue, 50% glycerol). To quantify the amount of decatenated DNA, photographic negatives of the ethidium-bromide-stained agarose gels were densitometrically scanned.

Immunoblots of topo IIα and IIβ protein

Cellular extracts from MKN and MKN/ADR cells were electrophoresed on 7% polyacrylamide gels and electroblotted onto Hybond-ECL membranes (Amersham). Topo IIα was detected with rabbit antibody raised against a synthetic peptide derived from the carboxyl terminal region of human topo IIα (TopoGEN, Columbus, Ohio). Topo IIβ was detected with affinity-purified rabbit antisera raised against a synthetic peptide corresponding to a unique region of the topo IIβ isoform (kindly provided by Dr. A. Kikuchi, Mitsubishi Kasei Institute of Life Science, Tokyo, Japan). The immunoblot signals were visualized by enhanced chemiluminescence (Amersham). The resulting X-ray films were scanned by densitometry to estimate the relative levels of topo IIα and IIβ protein present in each cell line (Biomed Instrument, Inc.). Immunoblots were carried out on three independent cellular extracts.

Detection and isolation of topo II-DNA complexes in vivo

Topoisomerase II-DNA covalent complexes were trapped as previously described [18]. In brief, MKN and MKN/ADR cells were left untreated or were treated with etoposide (100 μ*M*) for 30 min at 37 °C in serum-free RPMI medium (1×10^7 cells/ml). The medium was then removed and cells were directly lysed by the addition of lysis solution to give a final concentration of 1% Sarkosyl (in 10 m*M* TRIS-HCl, 1 m*M* EDTA). The lysates were overlayed onto CsCl step gradients and centrifuged at 30 000 rpm for 24 h at 20 °C with an SW40 rotor as described elsewhere [18]. Fractions (1 ml each) were collected from the bottom and the DNA peak was localized by measurement of the absorbance at 260 nm. Each fraction (50 μl) was mixed with 0.4 ml 25 m*M* sodium phosphate buffer (pH 6.5) and applied to a Hybond-ECL membrane (Amersham) in a slot-blot manifold. Each sample well was washed with 0.4 ml 25 m*M* sodium phosphate buffer (pH 6.5), after which the filter was air-dried. The membranes were incubated with affinity-purified antibody specific for the topo IIα or topo IIβ isoform as described above.

Northern-blot analyses of topo IIα and IIβ mRNA

Total RNA was isolated from exponentially growing MKN and MKN/ADR cells using Tri reagent (Molecular Research Center). RNA samples (10 μg/lane) were separated on 1% formaldehyde-agarose gel and vacuum-transferred to Hybond N^+ membranes (Amersham) with 20 × SSC (150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0). After fixation of RNA to membranes by alkaline treatment, the membranes were prehybridized at 65 °C in hybridization buffer [2 × SSPE, 7% SDS, 10% polyethylene glycol (PEG), 100 μg denatured herring-sperm DNA/ml]. Topo IIα mRNA and topo IIβ mRNA were detected with cloned topo IIα cDNA (3.03-kb EcoRI fragment) and topo IIβ cDNA (1.8-kb EcoRI-PstI fragment), respectively. All cDNA probes were labeled with [α-^{32}P]-deoxycytidine triphosphate ([α-^{32}P]-dCTP, Amersham) to a specific activity of 1×10^8 cpm/μg using a random-primer DNA labeling system (Amersham). Blots were hybridized with the individual probes at 65 °C for 12–18 h. Blots were washed twice with 2 × SSC/0.1% SDS for 15 min, twice with 1 × SSC/0.1% SDS for 15 min, and once with 0.1 × SSC/0.1% SDS for 30 min. The relative intensities of the bands were determined by videodensitometry (Biomed Instrument, Inc.).

Results

Sensitivity to various chemotherapeutic agents

The sensitivity of the two cell lines (MKN and MKN/ADR) to different chemotherapeutic agents is shown in Table 1. A higher level of resistance in MKN/ADR cells was observed for the topo II-targeted drugs, i.e., Adriamycin (11-fold), mitoxantrone (11-fold), and VP-16 (3-fold). Such large differences in sensitivity between the two cell lines were not observed for the agents whose mechanism of action does not directly involve topo II.

Cell growth rates

Since the sensitivity to topo II-targeted drugs could be influenced by differences in the growth rate, we determined the doubling times for the MKN and the MKN/ADR cell lines. The doubling time of the MKN/ADR cell line (2.1 days) was only slightly different from that of the MKN line (1.7 days; Table 2). We also measured the plating efficiency for both cell lines (see Materials and methods). The MKN/ADR cell line displayed no

Table 1 IC_{50} values and relative resistance to various chemotherapeutic agents as determined in the MKN cell line and the MKN/ADR subline (*MKN* Human stomach-adenocarcinoma cell line, *MKN/ADR* Adriamycin-resistant cell line, *CDDP* cisplatin, *CBDCA* carboplatin, *ADR* Adriamycin, *THP* THP-Adriamycin, *MMC* mitomycin C, *5-FU* 5-fluorouracil, *VP-16* etoposide, *Mitoxan* mitoxantrone)

Drugs	IC_{50} (μg/ml)[a]		RR[b]
	MKN	MKN/ADR	
CDDP	0.813 ± 0.273	0.287 ± 0.124	0.32
CBDCA	8.833 ± 2.597	9.533 ± 3.689	1.08
ADR	0.130 ± 0.021	1.413 ± 0.195	10.87
THP	0.136 ± 0.078	0.065 ± 0.006	0.48
MMC	0.024 ± 0.004	0.021 ± 0.003	0.87
5-FU	0.332 ± 0.082	0.410 ± 0.159	1.23
VP-16	8.666 ± 2.392	27.833 ± 0.850	3.21
Mitoxan	0.008 ± 0.000	0.085 ± 0.088	10.58

[a] IC_{50} values were evaluated by MTT assay, and results are presented as mean values ± SD for 3 independent experiments
[b] Relative resistance: IC_{50} of MKN/ADR/IC_{50} of MKN-45

Table 2 Plating efficiency obtained with 14-day incubation and doubling time as determined in the MKN cell line and the MKN/ADR subline

Cell lines	Number of cells plated	Number of colonies formed[a]	Plating efficiency (%)[b]	Doubling time (days)
MKN	1×10^3	87 ± 2.7	8.7	1.7
MKN/ADR	1×10^3	141 ± 6.0	14.1	2.1

[a] Mean value ± SD for numbers of colonies formed ($n = 3$)
[b] Plating efficiency (%): number of colonies formed/number of cells plated × 100

significant difference in plating efficiency as compared with the parental MKN line (Table 2).

Expression of drug-resistance-related genes

Multidrug resistance (MDR) may be caused by decreased drug accumulation mediated by the *mdr*-1 gene-encoded P-glycoprotein, which is responsible for drug efflux [17]. However, the cross-resistance pattern of the MKN/ADR cell line (Table 1) does not fit that of a typical MDR phenotype. To obtain direct evidence that the MDR phenotype was not expressed in the MKN/ADR cell line, we analyzed P-glycoprotein expression in both cell lines. For the analysis of MDR-1, cells were labeled with [^{35}S]-methionine at 50 μCi/ml for 16 h. The membrane proteins were then incubated with the antibody to MDR-1 (Centocor, Malvern, Pa.), immunoprecipitated with protein A Sepharose 4B, and resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 1A, there was no evidence of induction of P-glycoprotein in the MKN/ADR cell line. Furthermore, for investigation as to whether the expression of other drug-resistance-related proteins might be induced during the development of Adriamycin resistance in MKN/ADR cells, expression levels of GST-π (Fig. 1B) and GST-L (Fig. 1C) were examined by immunoblot analysis and SOD activity was measured (data not shown) [29]. However, no difference in these proteins was detected between MKN and MKN/ADR cells.

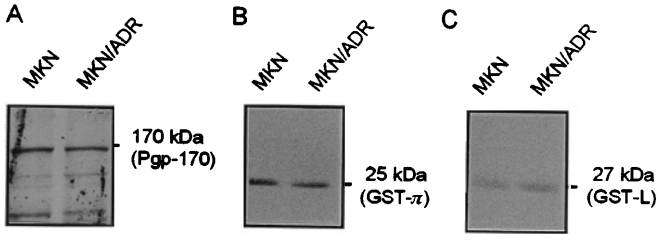

Fig. 1 Immunoprecipitation of MDR-1 and immunoblot analysis of GST-π and GST in MKN and MKN/ADR cells. **A** For the analysis of MDR-1 the membrane proteins were incubated with the antibody to MDR-1 and precipitated with protein A Sepharose 4B. For the analysis of **B** GST-π and **C** GST-L the cellular extracts were resolved by 10% SDS-PAGE, transferred to the nitrocellulose membrane, and detected with their specific polyclonal antibodies. Estimated molecular weights of MDR-1, GST-π, and GST-L are denoted in kDa

Topo I and topo II catalytic activity

Prominent resistance of MKN/ADR cells to topo II-targeted drugs suggested an alteration in topo II activity in resistant cells. We therefore assayed topo II activity in nuclear extracts of the MKN and MKN/ADR cells by the decatenation of kDNA. Topo II decatenation activity (per identical amounts of nuclear extract proteins) was approximately 3-fold lower in MKN/ADR extracts as compared with MKN extracts as determined by comparison of the banding intensities of the minicircles in several dilutions (Fig. 2). To determine if the reduced topo II activity in MKN/ADR cells reflected a generalized phenomenon of altered gene expression following cellular drug exposure, we compared the catalytic activity of the related nuclear enzyme DNA topo I in the drug-sensitive and drug-resistant cell types. Topo I activity was measured by the relaxation of the supercoiled plasmid in the absence of ATP. As can be seen in Fig. 3, topo I catalytic activities were equivalent in the MKN and MKN/ADR cells.

Topo IIα and IIβ protein levels

Reduced topo II levels could explain the observed Adriamycin resistance [6]; thus, we determined immunoreactive topo II levels in cellular extracts of log-phase

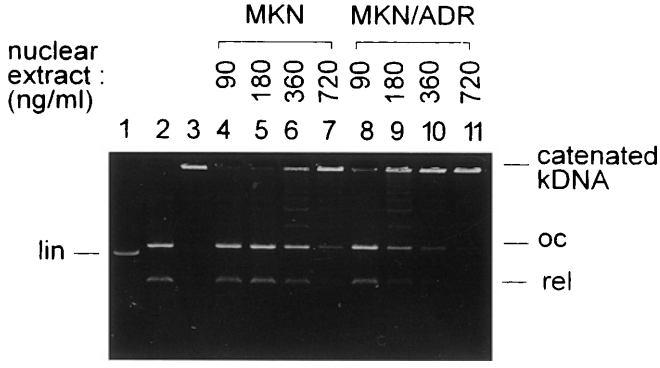

Fig. 2 Topo II activity detected in nuclear extracts from MKN and MKN/ADR cells. Topo II activity in nuclear extracts from MKN (*lanes 4–7*) and MKN/ADR cells (*lanes 8–11*) was measured by the decatenation assay of kDNA as described in Materials and methods. Reaction mixtures were incubated in the presence of various dilutions of nuclear extracts as shown. The extract protein amounts added from two cell lines are presented (*Lane 1* Xho I-digested linear kDNA, *lane 2* decatenated kDNA, *lane 3* catenated kDNA, *oc* nicked minicircles, *rel* relaxed minicircles)

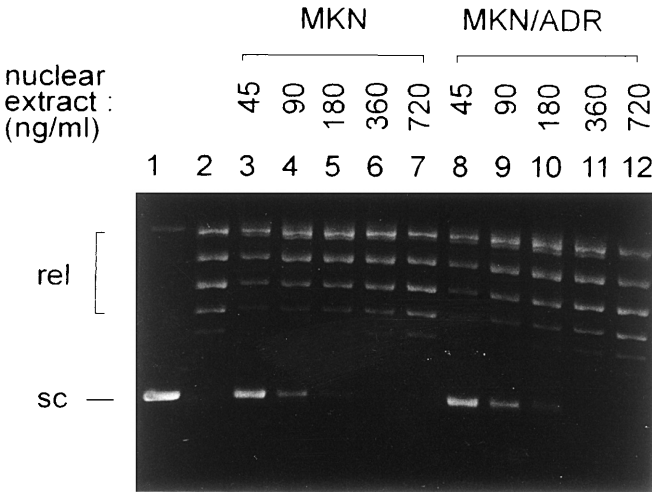

Fig. 3 Topo I activity detected in nuclear extracts from MKN and MKN/ADR cells. Topo I activity assays were performed by relaxation of supercoiled pBS as described in Materials and methods. Reaction mixtures were incubated in the presence of various dilutions of nuclear extracts from MKN (*lanes 3–7*) or MKN/ADR cells (*lanes 8–12*). The extract protein amounts added from two cell lines are presented (Lane 1 Supercoiled DNA only, *lane 2* relaxed DNA, *rel* relaxed plasmid, *sc* supercoiled DNA)

MKN and MKN/ADR cells in three independent experiments (Fig. 4). The relative polypeptide levels of topo IIα or IIβ were measured by quantitation of banding intensities detected with affinity-purified antibodies specific for each isoform on Western blots. Densitometric analysis of the autoradiographs revealed that topo IIα polypeptide levels in MKN/ADR cells were approximately 20-fold lower in the Adriamycin-resistant MKN/ADR cells than in the parental MKN cells (Fig. 4B). In contrast, topo IIβ levels were the same in these two cell lines (Fig. 4C). Additionally, topo I levels were identical in both lines (Fig. 4D).

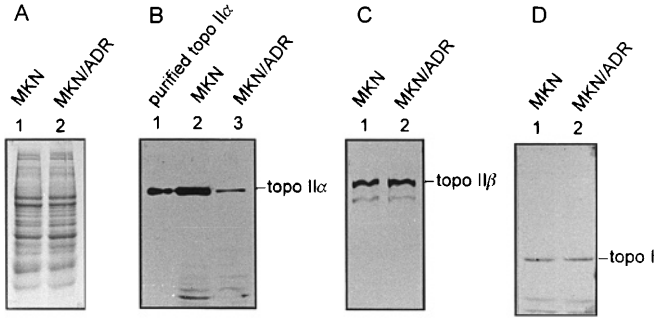

Fig. 4A–D Western-blot analysis of topo IIα and IIβ in cellular extracts of MKN and MKN/ADR cells. The same amounts of cellular extracts from MKN and MKN/ADR cells (120 μg/lane) were separated on 7% SDS-PAGE and transferred onto Hybond-ECL membranes. The blots were incubated with topo II isoform-specific antibodies. **A** Coomassie staining of cellular extracts separated by SDS-PAGE. **B** Topo IIα was detected with affinity purified rabbit anti-topo IIα-specific antibody, which recognizes the M_r 170 000 form of the topo II enzyme. **C** Topo IIβ was detected with topo IIβ-specific monoclonal antibody, which is specific for the M_r 180 000 enzyme form. **D** Topo I was detected with rabbit anti-topo I antibody

Formation of topo II-DNA cleavable complexes in MKN and MKN/ADR cells

To examine whether the reduced levels of topo IIα in resistant cells were proportional to the formation of in vivo topo IIα-DNA cleavable complexes, the cleavable complex formation in vivo in MKN and MKN/ADR cells was measured using the in vivo complexes of Enzyme (ICE) bioassay [18]. Topo II engages DNA in a cycle of single- and double-strand DNA breaks followed by rapid religation [19]. In the presence of topo II poisons such as etoposide a covalent topo II/DNA complex can be stabilized and trapped upon the addition of protein denaturants [13, 19]. Genomic DNA was purified using a preparative-step CsCl from cells that were left untreated or treated with etoposide. Gradient fractions were probed with antibodies to topo IIα or topo IIβ to determine the association with genomic DNA. As shown in Fig. 5, both isoforms were detected in the DNA peak fractions of cells treated with etoposide. Quantitation of the slot-blot signal with topo IIα-specific antibody revealed that cleavable complexes between endogenous topo IIα and DNA were formed at approximately 20-fold lower levels in the drug-resistant MKN/ADR cells as compared with the parental MKN cells (Fig. 5A). In contrast, endogenous topo IIβ was shown to form nearly identical amounts of cleavable complexes in the two cell lines (Fig. 5B). These results

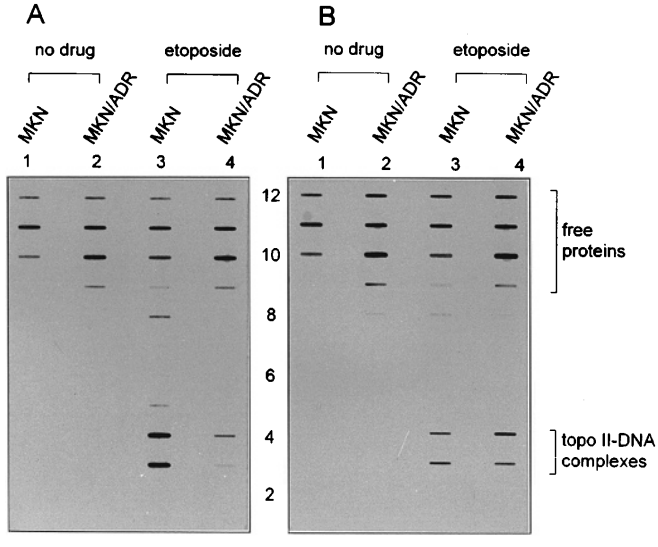

Fig. 5A,B Formation of endogenous topo II-DNA cleavable complexes. MKN (*lanes 1*, 3) and MKN/ADR cells (*lanes 2*, *4*) were treated with 100 μ*M* etoposide for 30 min and lysed with 1% Sarkosyl. Lysates were directly loaded onto a CsCl step gradient as described in Materials and methods. The gradient fractions were analyzed by immunoblotting with anti-topo IIα (**A**) or IIβ antibody (**B**). (*Lanes 1*, *2* no drug, *lanes 3*, *4* treatment with 100 μ*M* etoposide for 30 min followed by direct lysis in Sarkosyl)

indicate that the reduced level of topo IIα directly correlates with a reduction in in vivo drug-induced cleavable complex formation in the drug-resistant MKN/ADR cells.

Cloning and characterization of the topo IIα promoter region

To examine the possibility that changes in the *cis*-acting element in the topo IIα promoter might confer the transcriptional repression, we cloned and sequenced the promoter region of the topo IIα gene between −553 and +94 from the transcription start site in both cell lines. We did not detect any mutation in the promoter region of the topo IIα gene in the resistant cell line as compared with the sensitive cell line. The promoter sequences we determined were shown to be exactly the same with the previously reported human topo IIα promoter [11].

Topo IIα and IIβ mRNA levels

As reduced transcription or processing levels could be responsible for the observed reduction in topo II protein expression [9], we determined stable topo IIα and IIβ mRNA levels in the drug-sensitive and drug-resistant cell lines using isoform-specific cDNA probes. The radiolabeled topo IIα cDNA (3.03-kb EcoRI fragment) hybridized with a ~6.3-kb mRNA in both cell lines, and levels of the RNA transcript detected in MKN/ADR cells were nearly identical to those found in the parental MKN cells (Fig. 6A). The radiolabeled topo IIβ cDNA (1.8-kb EcoRI-PstI fragment) also hybridized with a prominent ~6.3-kb transcript in both cell lines. There was no significant difference in the amount of topo IIβ transcript from the MKN and MKN/ADR cells (Fig. 6A). Ethidium bromide staining of the gel was used to compensate for sample loading (Fig. 6B).

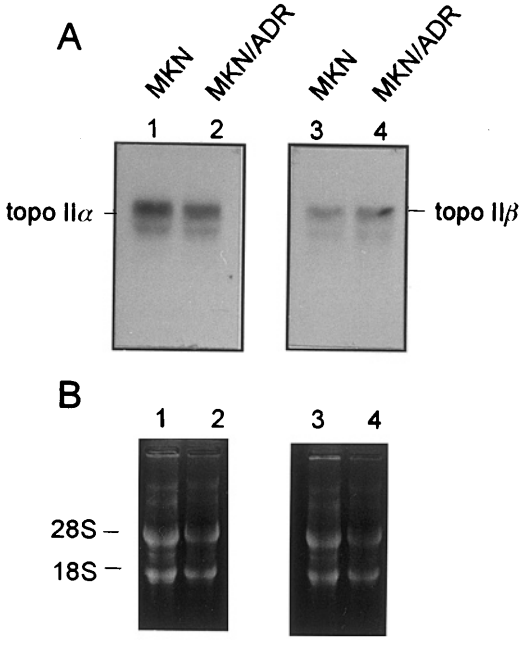

Fig. 6A,B Northern-blot analysis of topo IIα and IIβ expression in MKN and MKN/ADR cells. **A** Total RNA (10 μg/lane) was prepared from MKN and MKN/ADR cells, electrophoresed through 1% formaldehyde/agarose gels, and blotted onto Hybond N^+ membranes. The blot was probed with ^{32}P-labeled cDNA specific for topo IIα (*lanes 1*, *2*) or topo IIβ (*lanes 3*, *4*) **B** Ethidium bromide staining of agarose gel. The amount of RNA in each lane was monitored using 28S and 18S rRNA

Discussion

In many Adriamycin-resistant cell lines the cellular content of topo II appears to be a major determinant of the sensitivity to topo II-targeted agents [6]. MKN/ADR cells were also cross-resistant to mitoxantrone and VP-16, although relative levels of resistance were different (Table 1). Some mammalian cell lines displaying relatively specific resistance to topo II-targeted drugs have been described that appear to be resistant primarily due to a decrease in topo II levels [10]. Overall, the data suggest that the amounts of topo II protein present correlate closely with drug sensitivity. In other resistant cell lines the amount of immunoreactive topo II protein remains unchanged, but point mutations in the putative ATP-binding region and the active-site tyrosine region of topo II have been detected [5].

In this work we isolated an Adriamycin-resistant cell subline, MKN/ADR, from a human stomach-adenocarcinoma cell line by stepwise selection of the parental MKN cell line. Since P-glycoprotein overexpression was not detected in drug-resistant cells (Fig. 1), resistance to Adriamycin in MKN/ADR cannot be ascribed to MDR. This idea is further supported by the observation that the cross-resistance pattern of MKN/ADR cells was not similar to that expected for MDR as shown in Table 1. Adriamycin can also influence free radical formation [15]. Indeed, evidence has been presented that describes differential formation of hydroxyl radical formation by Adriamycin in sensitive and resistant MCF-7 human breast tumor cells [21]; however, we found no induction of a key enzyme related to free radical detoxification in MKN/ADR cells.

The topo I and topo II catalytic activities were measured in nuclear extracts. Total topo II catalytic activity was reduced 3-fold in the drug-resistant cell line as measured by the decatenation of kDNA (Fig. 2), whereas topo I catalytic activity was almost identical in extracts from both sensitive and resistant cells (Fig. 3). The differences in topo II catalytic activity were not influenced by a difference in growth rate or a decrease in topo II activities in nuclear extracts from MKN/ADR cells, because the doubling time of the MKN/ADR cell line (2.1 days) was only slightly increased relative to that of the MKN cell line (1.7 days; Table 2). Resistance to Adriamycin is specifically associated with altered

expression of the topo IIα isozyme form. Topo IIα polypeptide levels were approximately 20-fold lower in Adriamycin-resistant MKN/ADR cells as compared with parental MKN cells as measured by Western blotting (Fig. 4). In contrast, topo IIβ protein levels were the same in the two lines. A quantitative ICE bioassay allowed us to compare in vivo topo II-DNA covalent complexes in the two cell lines [18]. In vivo covalent complexes are readily detected in the presence of etoposide, and formation of complexes relates directly to the amount of genomic DNA damaged by endogenous topo II. The results revealed that the level of active topo IIα was reduced approximately 20-fold in the resistant cells, whereas the level of topo IIβ remained unchanged (Fig. 5).

Since the rearrangement and hypermethylation of the topo IIα gene may be associated with reduced topo II enzyme expression in cells selected for resistance to topo II-targeted drugs [2, 14, 23], we evaluated chromosomal rearrangement and CpG methylation for topo II genes in the drug-sensitive and drug-resistant cell lines. The Southern-blotting data indicate that the reduced levels of topo IIα protein in the drug-resistant cells appear to be unrelated to chromosomal rearrangement or hypermethylation of the topo IIα gene (data not shown). In addition, Northern blots showed that stable mRNA levels (and sizes) were nearly identical in the sensitive and resistant cell lines (Fig. 6). Since the expression of mRNA was similar, it was not surprising that the topo IIα promoter elements in the parent and resistant lines were identical.

The discovery that topo IIα polypeptide levels are lower in MKN/ADR cells as compared with the parental line is consistent with the finding that total enzyme activity is reduced 3-fold in the resistant line. However, we noted a nearly 20-fold difference in polypeptide levels between these two lines. There are several possible interpretations of this finding. First, decatenation assays cannot distinguish between topo IIα and topo IIβ; therefore, both isoforms of topo II are being measured. This alone may explain the difference. In this case, the beta isoform may provide compensatory activity that we would expect to see as extractable decatenation activity. Moreover, the in vivo topo IIα action on the genome was also reduced by 20-fold (as measured by the ICE bioassay). It therefore appears that the endogenous cleavage and religation activity of the topo IIα isozyme is reduced to a level that is commensurate with the polypeptide levels (as measured by Western blotting). It is also possible that topo II in resistant lines may be posttranslationally modified such that even very low levels of the alpha isoform may be sufficient to provide essential activity [22]. For example, phosphorylation has been reported to activate topo II activity [7]. Thus, we cannot conclude that resistant lines rely on the beta isoform for complementation. Other possibilities exist, such as reduced translational efficiency of the message and/or an increase in ubiquitination, leading to more rapid degradation of the topo IIα polypeptide. Changes in translational efficiency or in topo IIα stability could be caused by point mutation(s); however, we have not yet sequenced the mutant gene in its entirety. These and additional studies will be required to resolve the basis for the Adriamycin resistance in this particular case.

Acknowledgements We thank Drs. T. H. Lee and J. R. Spitzner for critical comments on the work. This work was supported in part by research grants from Bioproducts Research Center of Yonsei University (project 95-K1-04 01-03-05-3) and Korea Science and Engineering Foundation (project 961-0503-029-2) to I.K.C. and by NIH grants (2P30CA16058, UO1-CA63185, and RO1 CA63653) to M.T.M.

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